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Hepatic Effects of a Methionine-Choline-Deficient Diet in Hepatocyte RXR α -null Mice

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Abstract

Retinoid X receptor- α (RXR α) is an obligate partner for several nuclear hormone receptors that regulate important physiological processes in the liver. In this study the impact of hepatocyte RXR α deficiency on methionine and choline deficient (MCD) diet-induced steatosis, oxidative stress, inflammation, and hepatic transporters gene expression were examined. The mRNA of sterol regulatory element-binding protein (SREBP)-regulated genes, important for lipid synthesis, were not altered in wild type (WT) mice, but were increased 2.0- to 5.4-fold in hepatocyte RXR α -null (H-RXR α -null) mice fed a MCD diet for 14 days. Furthermore, hepatic mRNAs and proteins essential for fatty acid β -oxidation were not altered in WT mice, but were decreased in the MCD diet-fed H-RXR α -null mice, resulting in increased hepatic free fatty acid levels. Cyp2e1 enzyme activity and lipid peroxide levels were induced only in MCD-fed WT mice. In contrast, hepatic mRNA levels of pro-inflammatory factors were increased only in H-RXR α -null mice fed the MCD diet. Hepatic uptake transporters Oatp1a1 and Oatp1b2 mRNA levels were decreased in WT mice fed the MCD diet, whereas the efflux transporter Mrp4 was increased. However, in the H-RXR α -null mice, the MCD diet only moderately decreased Oatp1a1 and induced both Oatp1a4 and Mrp4 gene expression. Whereas the MCD diet increased serum bile acid levels and alkaline phosphatase activity in both WT and H-RXR α -null mice, serum ALT levels were induced (2.9-fold) only in the H-RXR α -null mice. In conclusion, these data suggest a critical role for RXR α in hepatic fatty acid homeostasis and protection against MCD-induced hepatocyte injury.

Keywords

Nuclear receptors; Retinoid X receptor-alpha; Non-alcoholic steatohepatitis; sterol regulatory element binding protein; fatty liver; methionine and choline deficient diet; hepatic transporters

Introduction

Non-alcoholic steatohepatitis (NASH) is a chronic liver disease that has histological features comparable to alcohol-induced steatohepatitis (Ludwig *et al.*, 1980). Whereas the cellular and molecular mechanisms involved in the pathogenesis of NASH are not fully understood, the initial stage of the disease is characterized by fat accumulation (steatosis), especially

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triglycerides, in hepatocytes. NASH is seen in patients with the metabolic syndrome, obesity, hyperlipidemia, and type 2 diabetes mellitus (Ludwig *et al.*, 1980). Furthermore, steatosis is known to stimulate lipogenesis through decreased expression of the peroxisome proliferator-activated receptor α (PPAR α), a nuclear receptor crucial for fatty acid oxidation (Wan *et al.*, 1995). Compelling evidence suggests that impaired fatty acid oxidation is the cause of fatty acid accumulation and steatosis in liver (Fromenty and Pessayre, 1995; Bradbury, 2006).

Another major regulator of lipogenesis in the liver is the sterol regulatory element binding proteins (SREBPs), transcription factors known to have important roles in *de novo* fatty acid and triglyceride synthesis (Horton *et al.*, 2002). The three SREBP isoforms, SREBP-1a, SREBP-1c, and SREBP-2, have overlapping functions, and activate the expression of more than 30 genes involved in lipogenesis (Horton *et al.*, 2002). Insulin-induced increases in SREBP-1c mRNA lead to elevated fatty acid synthesis and fatty liver (Shimomura *et al.*, 1999). It has been suggested that SREBP activity is a key factor involved in lipid accumulation in nonalcoholic fatty liver disease (NAFLD), and that activation of PPAR α and AMP-activated protein kinase (AMPK), which can suppress SREBP activity, may serve as potential treatment option for NASH (Zhou *et al.*, 2001; Ahmed and Byrne, 2007).

Whereas the etiology of NASH may be multifactorial and complex, emerging evidence indicates that nuclear receptors are important in the pathogenesis of NASH (Caldwell *et al.*, 2001; Dai *et al.*, 2003; Ip *et al.*, 2004; George and Liddle, 2008). For instance, thiazolidinediones, the most promising drug family for treating NASH, activate the nuclear receptor PPAR γ , leading to enhanced insulin sensitization in NASH patients and thereby decreasing steatohepatitis (Caldwell *et al.*, 2001). Activation of PPAR γ also has anti-inflammatory effects (Jiang *et al.*, 1998). Furthermore, WY-14,643, a potent PPAR α agonist, decreases hepatic triglyceride concentrations and diminishes hepatotoxicity in an animal model of NASH (Ip *et al.*, 2004). Interestingly, PPAR isomers and other type II nuclear receptors require retinoid X receptor alpha (RXR α) as the obligate heterodimeric partner for transcription of their target genes (Ulven *et al.*, 1998).

Retinoids have profound anti-inflammatory effects (Nozaki *et al.*, 2006). The ability of retinoids to inhibit the production of cytokines is mediated by their nuclear receptors (Uchimura *et al.*, 2001). In mouse models of noninsulin-dependent diabetes mellitus and obesity, the RXR agonists, LG100268 and LG100324, function as insulin sensitizers and decrease hyperglycaemia, hypertriglyceridaemia, and hyperinsulinaemia (Mukherjee *et al.*, 1997; Liu *et al.*, 2000). Chronic administration of LG100268 and LG100324 to rats reduced food intake and body weight gain (Liu *et al.*, 2000). This suggests that retinoids and their receptors play a role in lipid homeostasis (Mukherjee *et al.*, 1997; Liu *et al.*, 2000; Thacher *et al.*, 2000). In a recent report, we indicated that hepatocyte RXR α -null (H-RXR α -null) mice exhibited enhanced liver injury in association with both increased hepatic free fatty acids and production of pro-inflammatory cytokines (Gyamfi *et al.*, 2008b). Furthermore, low serum retinol levels are associated with hepatocellular carcinoma in patients with chronic liver disease (Newsome *et al.*, 2000). To date, there are no reports on the role of RXR α , the most abundant among the three RXR isoforms (α , β , γ) in liver, in the development of NASH (Ulven *et al.*, 1998). We hypothesize that a compromise in the retinoids/RXR α signaling pathway has a significant negative impact on the pathogenesis of NASH.

The MCD diet is a standard nutritional rodent model for NASH that produces key features of human NASH, including steatosis, hepatic inflammation, and fibrosis (Weltman *et al.*, 1996; Ip *et al.*, 2004). Elevated levels of alkaline phosphatase (ALP), a biochemical marker for cholestasis, are seen in subjects with NASH (Sorrentino *et al.*, 2005). In rodent studies, reduced expression of canalicular transporters results in cholestasis (Trauner *et al.*, 1999). Furthermore, changes in the expression and localization of hepatic transporters have been reported in patients

with chronic cholestatic liver disease (Keitel *et al.*, 2005). In addition, increased expression of proinflammatory cytokines implicated in NASH can alter the expression of hepatic transporters leading to a cholestatic phenotype (Geier *et al.*, 2003). We previously reported that the MCD diet can induce cholestasis; however, limited data exist on the influence of the MCD diet on xenobiotic transporters (Lickteig *et al.*, 2007; Gyamfi *et al.*, 2008a).

In this study we used a MCD diet to treat WT and H-RXR α -null mice to investigate the role of hepatocyte RXR α in the pathogenesis of NAFLD. The results of this study indicate increased susceptibility of the H-RXR α -null mice to steatohepatitis induced by the MCD diet. Our findings show that impaired fatty acid oxidation and inflammatory factors, rather than factors involved in oxidative stress, play significant roles in the pathogenesis of NASH in H-RXR α -null mice. Furthermore, down-regulation of hepatic Oatp1a1 and Oatp1b2 as well as up-regulation of basolateral efflux transporter Mrp4 may be critical pathologic features of the MCD diet ingestion in addition to steatosis and fibrosis.

Materials and Methods

Animals

Mice carrying the RXR α mutation in hepatocytes have been described previously (Wan *et al.*, 2000). Age-matched WT (of mixed genetic background of C57/Bl/6, 129/SvEvTac, and DBA-2) and H-RXR α -null mice (10-12 weeks old) were used in all the experiments (Wan *et al.*, 2000; Wan *et al.*, 2003). The mice were housed in steel microisolator cages at 22°C with a 12-h/12-h, light/dark cycle. Due to the tendency of animals to reduce their solid food intake during administration of a MCD diet, mice were given a liquid diet. After 2 days of feeding mice a control liquid diet, mice were randomized into two dietary groups (n = 8), and fed either a methionine-choline sufficient (control) or a MCD diet for 14 days. The diets were purchased from DYETS Inc. (Bethlehem, PA). The MCD diet was similar to the control diet except for complete removal of methionine and choline. For the control diet, methionine was provided at 1.18 g/L and choline at 0.66 g/L. Protein was 18.9% calories, fat was 16.5% of calories, and 64.5% of calories were from carbohydrate.

Mice were given free access to feed. During the experimental period, individual body weights were recorded at the start and once a week thereafter. All procedures were conducted in accordance with the NIH Guidelines for the Care and Use of Laboratory Animals and were approved by the Kansas University Medical Center Institutional Animal Care and Use Committee. After 2 weeks of feeding, blood samples were obtained and centrifuged at 3000 rpm for 15 min to collect serum. Livers were rapidly excised, weighed and a portion snap-frozen in liquid nitrogen and maintained at -80°C, and later used for RNA extraction, lipid peroxidation (LPO) assay, or lipid extraction. The rest of the fresh liver tissue was immediately homogenized for isolation of microsomes and cytosol. A portion of each liver was fixed in 10% formalin for hematoxylin and eosin (H & E) staining.

Serum alanine aminotransferase (ALT), ALP, cholesterol, triglyceride, and bile acid levels

Serum was stored at -20°C and used to assay ALT and ALP activities, as well as cholesterol, triglyceride, and bile acid concentrations. Serum ALT was determined using Liquid ALT Reagent kit (Pointe Scientific Inc., Brussels, Belgium). Serum ALP activity was determined using ALP (Liquid) Reagent Set (Pointe Scientific Inc., Canton, MI). Serum cholesterol and triglyceride levels were determined by cholesterol E-test and triglyceride E-test kits (Wako Pure Chemical Industries, Richmond, VA), respectively. Serum bile acid concentrations were determined using a commercially available kit (Colorimetric Total Bile Acids Assay Kit; Bioquant, San Diego, CA).

H & E Staining of Liver Sections and morphology analysis

Following fixation of the livers with 10% formalin/phosphate-buffered saline, livers were sectioned and stained with H & E for histological examination. Slides were viewed blindly and scored for steatosis, inflammation, necrosis, and fibrosis using our previously described criteria (Dai *et al.*, 2003). The liver pathology was scored as follows: steatosis (the percentage of liver cells containing fat), <25% = 1+, <50% = 2+, <75% = 3+, >75% = 4+; inflammation, necrosis, and fibrosis, 1 focus = 1+, 2 or more foci = 2+.

Hepatic triglyceride, nonesterified fatty acid (NEFA), and thiobarbituric acid-reactants (TBARS) levels

Total liver lipids were extracted from 100 mg of liver homogenate using methanol and chloroform as previously described (Folch *et al.*, 1957; Zhou *et al.*, 2006). Hepatic triglyceride was quantified using a Triglyceride test kit (Wako pure Chemical Industries, Richmond, VA). Hepatic NEFA levels were determined using the NEFA C test kit (Wako Pure Chemical Industries, Richmond, VA). For determination of lipid peroxides, 50 mg of liver was homogenized in 1.15% KCl. TBARS were measured in 200 µl liver homogenate as described previously (Gyamfi and Wan, 2006).

Hepatic Cyp2e1 activity

Microsomal fractions were separated from fresh liver tissue as described previously (Gyamfi *et al.*, 2006). Cyp2e1 activity in liver microsomes was estimated colorimetrically by measuring the hydroxylation of *p*-nitrophenol to 4-nitrocatechol (Reinke and Moyer, 1985).

Western blot analysis

Cytosolic and microsomal fractions were extracted as described previously (Gyamfi *et al.*, 2006). Liver homogenate (50 µg/lane), microsomes (30 µg/lane) and cytosol (30 µg/lane) were separated by 10 or 15% SDS-PAGE gels, electroblotted onto polyvinylidene difluoride (PVDF) membranes and immunoblotted with anti-acyl-Coenzyme A oxidase 1 (ACOX1)(ABGENT, San Diego, CA), anti-interleukin-1β (IL-1β)(Chemicon International, Temecula, CA, USA) or anti-Cyp4a14 and anti-liver fatty acid binding protein (L-FABP)(Santa Cruz Biotechnology, Santa Cruz, CA, USA) antibody. Blots were then incubated with the appropriate peroxidase-conjugated anti-rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA, USA) diluted in Tris-buffered saline with 0.1% Tween 20 (TBST) plus 1% non-fat dry milk for 1 h at room temperature. Following probing, blots were stripped and reprobed with anti-β-actin (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-gapdh (Abcam Inc., Cambridge, MA, USA) antibody. Proteins were viewed using enhanced chemiluminescence. Protein concentrations were determined by the Bradford method (Bradford, 1976).

Quantification of mRNA levels using real-time polymerase chain reaction (PCR) and branched DNA signal amplification (bDNA) assay

Total RNA was isolated from frozen liver tissues using the TRIzol reagent according to the manufacturer's protocol (Invitrogen, Carlsbad, CA). RNA concentration and quality were determined spectrophotometrically at 260 nm and the A260/A280 ratio, respectively. Real-time PCR was used to quantify the mRNA level of macrophage inflammatory protein-2 (MIP-2), IL-1β, tumor necrosis factor α (TNFα), α-smooth muscle actin (α-SMA), L-FABP, carnitine palmitoyltransferase 1 (CPT-1), ACOX1, sterol regulatory element-binding protein (SREBP)-1c, SREBP-2, PPARα, HMG-CoA reductase, acetyl CoA carboxylase-1a (ACC-1a), fatty acid synthase (FAS), fatty acid translocase (FAT/CD36), Cyp4a14, Cyp7a1, Cyp8b1, β-actin, and Gapdh as described previously (Gyamfi *et al.*, 2008b). Primers and probes (Table 1) were designed using Primer Express 2.0 (Applied Biosystems, Foster City, CA). The amplification reactions were carried out in an ABI Prism 7900 sequence detection system

(Applied Biosystems, Foster City, CA) as described previously (Gyamfi *et al.*, 2008b). The mRNA of hepatic transporters was quantified using the bDNA assay (QuantiGene, High Volume bDNA Signal Amplification Kit; Panomics, Fremont, CA) with modifications (Hartley and Klaassen, 2000). Specific transporter probe sets were designed as previously described: Mrp2, 3, and 4 (Maher *et al.*, 2005); Ntcp, Bsep, Mdr2, and Mdr1b (Maher *et al.*, 2006); and organic anion transporting polypeptide (Oatp) 1a1, 1a4, 1b2 (Cheng *et al.*, 2005). All reagents for analysis (i.e., lysis buffer, amplifier/label probe buffer, and substrate solution) were supplied by the manufacturer (QuantiGene, High Volume bDNA Signal Amplification Kit; Panomics, Fremont, CA). Total RNA (1 µg/µl; 10 µl/well) was added to each well of a 96-well plate containing capture hybridization buffer and 50 µl of each diluted probe set. Total RNA was allowed to hybridize to each probe set overnight at 53°C. Subsequent hybridization steps were carried out per the manufacturer's protocol and luminescence was quantified with a Quantiplex 320 bDNA Luminometer interfaced with Quantiplex Data Management Software Version 5.02 for analysis of luminescence from 96-well plates. The luminescence for each well was reported as relative light units (RLU) per 10 µg total RNA.

Statistical Analysis

Data are presented as means ± S.E.M (n = 4-8) and analyzed using SigmaStat 3.5 software (Systat Software, Inc. Chicago, IL). Effect of genotype, MCD diet, and their interaction were analyzed using a two-way analysis of variance (ANOVA), with Bonferroni post-hoc tests. A *P* value of < 0.05 was considered statistically significant.

Results

Effect of the MCD diet and RXRα deficiency on body weight, liver weight serum and hepatic lipid levels, lipid peroxide levels, Cyp2e1 activity, steatosis, and inflammation

The MCD diet decreased the body weight in both the WT and H-RXRα-null mice (Table 2). However, the decrease in body weight was greater in H-RXRα-null mice than in WT mice (Table 2). The MCD diet also decreased liver weight in both genotypes of mice, however, a statistically significant difference was found only in H-RXRα-null mice (Table 2). When liver weight was expressed as percent of body weight, no significant change was found in both genotypes of mice fed the MCD diet (Table 2). The genotype influenced the serum cholesterol and triglycerides levels which were higher in H-RXRα-null mice than in WT mice (Table 2). The MCD diet decreased serum cholesterol and triglyceride levels in both genotypes of mice (Table 2). Two-way ANOVA revealed significant effects (*P* < 0.05) of genotype and the MCD diet on serum cholesterol and triglyceride levels. Hepatic triglyceride levels were increased by the MCD diet in both WT and in H-RXRα-null mice (Table 2). The basal hepatic NEFA levels between WT and H-RXRα-null mice were not different (Table 2). Furthermore, the MCD diet did not increase NEFA levels in WT mice, but it did in livers of the H-RXRα-null mice (Table 2). WT mice fed the MCD diet had increased hepatic LPO as quantified by the TBARS assay (Table 2). The H-RXRα-null mice not only had constitutively lower TBARS, the MCD diet did not increase TBARS in their livers (Table 2). Cyp2e1 is involved in LPO, and its levels are increased in a rat dietary model of steatohepatitis (Weltman *et al.*, 1996). Consistent with an increase in LPO in the MCD-fed WT mice, Cyp2e1 enzyme activity was increased (Table 2). This increase in Cyp2e1 activity was not observed in H-RXRα-null mice fed the MCD diet (Table 2). The increase in TBARS levels and Cyp2e1 activity by the MCD diet in WT mice was higher than that in H-RXRα-null mice (Table 2). To determine lipid accumulation in liver, H & E staining was conducted. Liver sections indicated that lipid droplets were rare in livers of mice fed the control diet. Irrespective of the genotype, lipid droplets were common in both WT and H-RXRα-null mice fed the MCD diet (Fig. 1). The histological score indicated the presence of steatosis in both genotypes of mice fed the MCD diet (Table 2 and Fig. 1). While

a mild inflammation was observed by histology score in only H-RXR α -null mice (Table 2), however, necrosis and fibrosis were absent in both genotypes of mice (data not shown).

Effect of the MCD diet and RXR α deficiency on mRNA levels of lipogenic genes

The basal mRNA level of SREBP-1c was not different between WT and H-RXR α -null mice (Fig. 2). Furthermore, the MCD diet did not significantly alter SREBP-1c gene expression in either WT or H-RXR α -null mice (Fig. 2). The MCD diet did not increase mRNA levels of FAS and ACC-1a, target genes for SREBP-1c in WT mice, whereas they were increased 4.8- and 2.0-fold, respectively, in H-RXR α -null mice administered the MCD diet (Fig. 2). Furthermore, the increase in mRNA levels of FAS and ACC-1a by the MCD diet in H-RXR α -null mice was higher than in WT mice fed the MCD diet (Fig. 2). Two-way ANOVA indicated that the increase in FAS and ACC-1a mRNA levels in the H-RXR α -null mice was influenced by the interaction of the genotype and the MCD diet. The mRNA levels of SREBP-2 were not different between the WT and H-RXR α -null mice fed the control diet (Fig. 2). Whereas the MCD diet did not alter SREBP-2 mRNA levels in the WT mice, it increased SREBP-2 mRNA levels 2.2-fold in H-RXR α -null mice (Fig. 2). The SREBP-2-target gene HMG CoA reductase was not significantly increased in the WT mice by the MCD diet. However, HMG CoA reductase mRNA levels were increased 5.4-fold in the H-RXR α -null mice fed the MCD diet (Fig. 2). Two-way ANOVA indicated that the increase in SREBP-2, but not HMG CoA mRNA levels in the H-RXR α -null mice was influenced by the combined effect of the genotype and the MCD diet. The MCD diet increased the free fatty acid uptake transporter CD36 3.7-fold in WT mice but not in H-RXR α -null mice (Fig. 2).

Effect of the MCD diet and RXR α deficiency on mRNA levels of fatty acid oxidation genes

Hepatocyte RXR α deficiency did not change basal PPAR α mRNA levels (Fig. 3). Furthermore, the MCD diet did not alter PPAR α mRNA levels in WT mice, but the MCD diet decreased PPAR α mRNA levels in H-RXR α -null mice (Fig. 3). The level of CPT-1 mRNA was similar between WT and H-RXR α -null mice (Fig. 3). The MCD diet did not change the CPT-1 gene expression in WT mice, however, the MCD diet markedly decreased CPT-1 gene expression in the H-RXR α -null mice (Fig. 3). Furthermore, both the constitutive ACOX1 mRNA and protein levels were lower in the H-RXR α -null mice than in WT mice (Fig. 3). The MCD diet did not affect either the ACOX1 mRNA or protein levels in WT mice, whereas both were decreased in the H-RXR α -null mice (Fig. 3). The basal expression of L-FABP mRNA and protein were lower in H-RXR α -null mice than in WT mice (Fig. 3). Furthermore, the MCD diet did not alter the L-FABP mRNA or protein levels in WT mice (Fig. 3). The H-RXR α -null mice had about 50% less L-FABP mRNA and protein than WT mice, and the MCD diet decreased it further in the H-RXR α -null mice (Fig. 3). Cyp4a14 mRNA and protein levels were much lower in H-RXR α -null mice than in WT mice (Fig. 3). The Cyp4a14 mRNA was increased 1.8-fold, but not the protein in WT mice fed the MCD diet. The MCD diet did not increase either Cyp4a14 mRNA or protein levels in the H-RXR α -null mice (Fig. 3) and the levels were lower compared to WT mice fed the MCD diet (Fig. 3).

Effect of the MCD diet and RXR α deficiency on mRNA and protein levels of pro-inflammatory genes

The MCD diet did not induce the levels of MIP-2, TNF α , IL-1 β , and α -SMA mRNA in the WT mice (Fig. 4). However, the MCD diet increased MIP-2, TNF α , IL-1 β , and α -SMA mRNA by 2.5-, 1.7-, 2.0-, and 1.7-fold, respectively, in H-RXR α -null mice (Fig. 4). The increase in MIP-2 and IL-1 β mRNA levels by the MCD diet in H-RXR α -null mice was higher than in WT mice fed the MCD diet (Fig. 4). Hepatocyte RXR α deficiency did not change basal IL-1 β mRNA levels but increased IL-1 β protein in H-RXR α -null mice (Fig. 4). The MCD diet did not increase IL-1 β protein levels in WT mice (Fig. 4). However, the MCD diet increased

IL-1 β protein levels in H-RXR α -null mice (Fig. 4), and this increase was higher than in WT mice (Fig. 4). Two-way ANOVA revealed that the increase in MIP-2, IL-1 β , and α -SMA mRNA levels in H-RXR α -null mice was influenced by the interaction of genotype and the MCD diet (Fig. 4).

Effect of the MCD diet and RXR α deficiency on mRNA levels of hepatic bile acid synthesis genes

Bile acids are the end products of hepatic cholesterol metabolism, and also act as signaling molecules (Chiang, 1998). Interestingly, changes in bile acid synthesizing genes have not been examined after feeding a MCD diet. We therefore examined whether feeding the MCD diet was associated with changes in Cyp7a1 gene expression, which catalyzes the rate-limiting step in bile acid formation from cholesterol and sterol 12 α -hydroxylase (Cyp8b1), required for the synthesis of cholic acid (Chiang, 1998). The MCD diet did not statistically decrease Cyp7a1 gene expression in WT mice, but decreased Cyp7a1 mRNA levels 50% in the H-RXR α -null mice (Fig. 5). The basal Cyp8b1 mRNA level was similar in the H-RXR α -null mice compared to WT mice (Fig. 5). The MCD diet decreased the mRNA levels of Cyp8b1 50 and 70% in WT and H-RXR α -null mice, respectively (Fig. 5).

Effect of the MCD diet and RXR α deficiency on mRNA levels of hepatic uptake and efflux transporter genes

Ntcp is responsible for the uptake of bile acids from portal blood into hepatocytes. The MCD diet did not affect the expression of Ntcp in either genotype (Fig. 6). However, the MCD diet decreased the mRNA of the hepatic uptake transporter Oatp1a1 90 and 60% in WT and H-RXR α -null mice, respectively (Fig. 6). While the MCD diet did not affect the Oatp1a4 mRNA levels in WT mice, the MCD diet interacted with the genotype to induce the Oatp1a4 gene 4-fold in the H-RXR α -null mice (Fig. 6). The increase in Oatp1a4 mRNA levels by the MCD diet was higher in the H-RXR α -null mice than in WT mice (Fig. 6). The MCD diet inhibited Oatp1b2 gene expression 60% in WT mice, but not significantly in H-RXR α -null mice (Fig. 6). Feeding the MCD diet did not change the expression of the canalicular efflux transporters Bsep, Mrp2, Mdr2, and Mdr1b in either genotype of mice (Fig. 7). However, the basal mRNA level of Mdr2, which transports phospholipids into bile, was less in the H-RXR α -null mice than in the WT mice (Fig. 7). The expression of basolateral efflux transporters was also quantified (Figure 8). The basal Mrp3 expression was much lower in the H-RXR α -null mice than in the WT mice, suggesting that RXR α regulates constitutive expression of Mrp3 as previously reported (Cherrington *et al.*, 2003). The MCD diet did not have a significant effect on Mrp3 expression in either genotype of mice (Fig. 8). However, Mrp4 expression was increased by the MCD diet in both WT (3.7-fold) and H-RXR α -null mice (2.5-fold) (Fig. 8).

Effect of the MCD diet on serum ALT, ALP, and bile acids

After two weeks on the MCD diet, serum ALT activity was measured as an index of hepatocyte injury. The MCD diet did not increase ALT levels in WT mice. However, the MCD diet increased ALT levels in H-RXR α -null mice (Fig. 9). Serum ALP levels were lower in the H-RXR α -null mice compared to WT mice (Fig. 9). The MCD diet increased serum ALP 70 and 100% in H-RXR α -null and WT mice, respectively (Fig. 9). Serum bile acids were increased 70% in both WT and H-RXR α -null mice fed the MCD diet (Fig. 9). Two-way ANOVA revealed significant effects ($P < 0.05$) of genotype and the MCD diet on ALT and ALP levels but not on serum bile acid levels (Fig. 9).

Discussion

The purpose of the current study was to examine the role of hepatocyte RXR α in the pathogenesis of NAFLD/NASH and how changes in expression of xenobiotic transporters

contribute to pathogenesis of NASH. To examine these, WT and H-RXR α -null mice were fed a liquid MCD diet for 2 weeks.

The mechanisms of progression of a simple fatty liver to a fatty liver with liver injury or inflammation (NASH) remain unclear. However, a two-hit progression has been suggested (Day and James, 1998). Accumulation of fat in hepatocytes has been described as the “first hit” that serves as a potent stimulus for the “second hit”, including *S*-adenosylmethionine depletion, lipid toxicity, mitochondrial dysfunction, oxidative stress, apoptosis, and inflammation, culminating in liver injury (Day and James, 1998; Wortham *et al.*, 2008). The mechanisms of steatosis development in the MCD model involve limiting the availability of methionine and choline which are important precursors for phosphatidylcholine synthesis (Vance and Vance, 1985). This in turn inhibits very low density lipoprotein (VLDL) assembly and blocks triglycerides and free fatty acids secretion from hepatocytes (Vance and Vance, 1985). Whether the MCD diet can also induce lipogenic genes has not been fully explored. Subsequent experiments therefore characterized lipid accumulation and expression of lipogenic genes.

Feeding the MCD diet increased steatosis in both WT and H-RXR α -null mice indicating that steatosis was promoted by methionine and choline deficiency. Contrary to expectation, lipid accumulation and hepatic triglyceride levels were similar after the MCD diet between WT mice and H-RXR α -null mice (Fig. 1 and Table 2), suggesting that absence of RXR α in hepatocytes did not affect lipid accumulation in this model. We next sought to explore how the MCD diet ingestion and RXR α deficiency affected expression of genes involved in lipid synthesis.

The ingestion of the MCD diet was associated with increased expression of several hepatic lipogenic genes known to be controlled by SREBP-1c and SREBP-2 (Horton *et al.*, 2002) (Fig. 2). The SREBP-regulated battery of genes, including ACC-1a, FAS, and HMGCoA reductase was increased by the MCD diet in H-RXR α -null mice, but not in WT mice. In contrast, the effect of MCD diet on triglyceride accumulation in the WT mice appears to involve CD36, rather than SREBP-1- and SREBP-2-regulated genes, because CD36 mRNA levels were increased only in the WT mice (Fig 2). To date, this is the first report to demonstrate induction of SREBP-target genes while consuming a MCD diet. Interestingly, the induction of the SREBP-regulated genes was observed after a combination of MCD and high fat diet were fed to rats (Ota *et al.*, 2007). Furthermore, SREBP-1c is activated by insulin and glucose leading to elevated mRNA levels of its target genes (Shimomura *et al.*, 1999). It is possible that ingestion of the MCD diet by the H-RXR α -null mice could lead to hyperinsulinemia and induction of the SREBP-target genes. As human NASH typically occurs in obesity and insulin resistance, the transcriptional regulation of the SREBPs by the MCD diet in H-RXR α -null mice is important and remains to be investigated (Ludwig *et al.*, 1980; Ahmed and Byrne, 2007).

Another mechanism by which the MCD diet stimulates steatosis is inhibition of fatty acid oxidation (Koteish and Diehl, 2001). Furthermore, it has been demonstrated that activation of genes involved in fatty acid oxidation by the PPAR α agonist WY-14,643 ameliorated MCD-induced steatohepatitis (Ip *et al.*, 2003). CPT-1 mRNA levels as well as ACOX1 and L-FABP mRNA and protein levels were reduced in the MCD-fed H-RXR α -null mice, and such reductions were not found in the WT mice (Fig. 3). CPT-1 controls the rate of mitochondrial β -oxidation and regulates the deposition or oxidation of fatty acids in the liver (Zammit, 1999). However, ACOX1 is the first and rate-limiting enzyme in peroxisomal β -oxidation encoding the enzymes responsible for the transfer of shortened fatty acids from peroxisomes to mitochondria (George and Liddle, 2008). L-FABP enhances hepatic uptake of fatty acids, exposing them to the fatty acid oxidation machinery (Desvergne *et al.*, 1998). Furthermore, fatty acids are also oxidized through the microsomal ω -oxidation system by Cyp4a enzymes (Reddy and Hashimoto, 2001). The basal Cyp4a14 mRNA and protein levels were significantly

reduced and were not induced after ingestion of the MCD diet in the H-RXR α -null mice (Fig. 3). Based on the decrease in mRNAs critical for free fatty acid metabolism in the livers of the H-RXR α -null mice, fatty acid accumulation was predicted. Indeed, free fatty acid levels were increased in the MCD-fed H-RXR α -null, but not in WT mice (Table 2). Intracellular accumulation of free fatty acids may damage cell membranes and possibly contribute to hepatocyte injury. Supportive of our findings, decreased expression of genes encoding enzymes for peroxisomal and mitochondrial β -oxidation were also observed in mice made vitamin A deficient by feeding pregnant female mice from day 10 of gestation and their pups vitamin A deficient diets for 9 weeks (Kang et al., 2007). Overall, our data indicate that both vitamin A and its receptor RXR α are important for proper fatty acid homeostasis in the liver.

The present data also indicated that RXR α deficiency did not further enhance lipid accumulation in this experimental NASH model. Many factors involved in lipid homeostasis were altered during the MCD diet treatment because of RXR α deficiency. For example, PPAR α -mediated fatty acid oxidation is compromised in hepatocyte RXR α deficient mice; the expression of CD36 is up-regulated in MCD-treated WT mice, but not in MCD-treated H-RXR α -null mice; and SREBP-mediated genes are up-regulated in MCD-treated H-RXR α -null mice, but not in MCD-treated WT mice. These variables might eventually counter balance each other and result in no difference in the degree of steatosis in MCD-treated WT and H-RXR α -null mice.

Products of oxidative stress including lipid peroxides and cytokines have been found in patients with NASH suggesting their involvement in the transition from steatosis to steatohepatitis (Crespo et al., 2001; Reid, 2001). The impaired fatty acid oxidation and the resultant increase in hepatic NEFA levels suggested that the MCD diet could induce LPO in the H-RXR α -null mice. Contrary to expectation, we observed increased LPO only in the WT mice but not in H-RXR α -null mice (Table 2). As expected, Cyp2e1 enzyme activity was induced in the MCD-fed WT mice (Table 2). Consistent with the lack of LPO, Cyp2e1 enzyme activity and Cyp4a14 gene and protein expression were not increased in the MCD-fed H-RXR α -null mice (Fig. 3). Previous reports have shown that the MCD diet or a high fat diet can cause liver injury without induction of LPO and Cyp2e1 indicating that the extent of LPO does not always predict degree of toxicity (Deng et al., 2005; Arsov et al., 2006). The MCD diet produces inflammation (Ip et al., 2003). In agreement with these observations, gene expression of pro-inflammatory cytokines and chemokines were activated in the MCD diet-fed H-RXR α -null mice, but not in WT mice (Figs. 4). Taken together, our observation that the H-RXR α -null mice developed liver injury without both increased LPO and induction of Cyp2e1, but rather with elevated levels of pro-inflammatory cytokines, chemokines with mild histologic inflammation, suggests that oxidative stress is not an early event in the progression of steatosis to steatohepatitis as seen by other investigators (Sahai et al., 2004; Sundaram et al., 2005). The current study was done using mice treated with the MCD diet for 2 weeks, which is relatively a short treatment. It is therefore possible to speculate that oxidative stress is delayed in H-RXR α -null mice fed the MCD diet.

Expression of bile acid synthesis and hepatic transporter genes was quantified in an attempt to reveal further changes induced by the MCD diet in the absence of RXR α in hepatocytes. Moreover, the farnesoid X receptor (FXR), a key regulator of bile acid, lipid, and glucose metabolism, heterodimerizes with RXR α (Blumberg and Evans, 1998; Fiorucci et al., 2007). In the current study, the MCD diet resulted in significant suppression of key bile acid synthesizing genes Cyp8b1 and Cyp7a1 mRNA levels (Fig. 5). Interestingly, both hepatic Cyp7a1 and Cyp8b1 mRNA levels were reduced in MCD-fed H-RXR α -null mice suggesting that the regulation of these genes is not impaired in the absence of RXR α . Previous reports indicate that repression of Oatp1a1 is completely lost in PXR- and CAR-null mice after bile-

duct ligation (BDL) (Stedman *et al.*, 2005). It was of interest to examine the effects of the MCD diet on hepatic uptake transporters.

The mRNA levels of both Oatp1a1 and Oatp1b2 were markedly decreased but Oatp1a4 was unaffected by the MCD diet in WT mice (Fig. 6). The MCD diet also decreased Oatp1a1 mRNA levels in the H-RXR α -null mice; however, the decrease was not as pronounced as that seen in the WT mice. Cytokines including TNF α , IL-6, and IL-1 β have been implicated as important in down-regulation of hepatic transporters during cholestasis (Hartmann *et al.*, 2002; Geier *et al.*, 2003). The reduced MCD-induced suppression of Oatp1a1 expression in the H-RXR α -null mice suggests the co-dependence of PXR and CAR on RXR α in Oatp1a1 down-regulation. Contrary to expectation, the MCD diet markedly induced Oatp1a4 without any effect on Oatp1b2 in H-RXR α -null mice. This data is similar to a previous report in which the bile duct ligation (BDL) model was used to induce cholestasis resulting in Oatp1a4 gene induction with inhibition of Oatp1a1 and Oatp1b2 (Slitt *et al.*, 2007). Furthermore, the hepatotoxicants, acetaminophen and carbon tetrachloride can increase Oatp1a4 mRNA levels (Aleksunes *et al.*, 2005). A possible explanation is that, the Oatp1a4 induction seen in H-RXR α -null mice fed the MCD diet may increase bile acid transport into hepatocytes for bile acid hydroxylation and detoxification. In addition, Oatp1a4 can function bidirectionally (Li *et al.*, 2000). Thus, induction of Oatp1a4 may assist in bile acid efflux similar to Mrp3 and Mrp4. However, liver injury was observed in H-RXR α -null mice fed the MCD diet, suggesting that Oatp1a4 induction was not sufficient to ameliorate MCD-induced hepatotoxicity.

Reports indicate that induction of Bsep, Ost β , and Mrp-isoforms in cholestatic mice is an adaptive mechanism to enhance excretion of toxic bile acids and conjugates into bile and/or portal blood (Fiorucci *et al.*, 2007). Mrp3 and Mrp4 can transport bile acids (Mennone *et al.*, 2006; Fiorucci *et al.*, 2007). The induction of Mrp3 and Mrp4 by the MCD diet has been reported in rats (Lickteig *et al.*, 2007). In the present study, while basal Mrp3 mRNA expression was lower in H-RXR α -null mice, the Mrp3 expression in both genotypes of mice was not induced after feeding the MCD diet (Fig. 8). However, the MCD diet markedly induced Mrp4 mRNA expression in WT, but was only moderately increased in the H-RXR α -null mice. The induction of Mrp4 seen in this study is also similar to that seen in bile duct-ligated mice, and lack of Mrp4 induction in Mrp4-null mice led to impaired cytoprotection (Mennone *et al.*, 2006). FXR, PXR, and CAR regulate Mrp4 gene expression (Fiorucci *et al.*, 2007). Bile-duct obstruction in FXR-null mice causes a robust induction of Mrp4 in the basolateral membrane leading to less liver injury compared to WT mice (Stedman *et al.*, 2006). Because Mrp4 mRNA induction by MCD was observed in both WT and H-RXR α -null mice, this finding indicates that MCD-induced Mrp4 expression may be mediated through an RXR α -independent mechanism. Indeed, in a recent report, the treatment of WT and nuclear factor E2-related factor-2 (Nrf2)-null mice with Nrf2-activating chemicals, oltipraz and butylated hydroxyanisole, demonstrated that the induction of Mrp4 is Nrf2-dependent (Maher *et al.*, 2007). This suggests that Nrf2 may be responsible for Mrp4 induction in the H-RXR α -null mice fed the MCD diet. Taken together, compared to WT mice, these data indicate that hepatocyte RXR α deficiency does not lead to significant impairment in bile acid homeostasis during MCD diet administration.

The above changes observed after the MCD diet in oxidative stress and expression of genes involved in fatty acid oxidation, pro-inflammatory cytokines, bile acid synthesis, and hepatic transporters prompted us to examine markers for liver injury in WT and H-RXR α -null mice. While the MCD diet caused hepatocyte injury only in H-RXR α -null mice seen as elevated ALT levels, it significantly increased serum bile acid levels and ALP activity in both WT and H-RXR α -null mice (Fig. 9). Serum bile acids and ALP are markers of cholestasis. Our findings indicate that the MCD diet caused cholestasis in both genotypes of mice. It is possible that the cholestasis observed in mice fed the MCD diet in this study might be due to impaired ductular

permeability and compression or obstruction of the bile ducts by the accumulated fat in hepatocytes.

Collectively, these data indicate that deficient expression of RXR α in hepatocytes induces a disturbed balance between fatty acid synthesis and oxidation when consuming a MCD diet, ultimately, favoring fatty acid accumulation, inflammation, and liver injury in H-RXR α -null mice, as shown in Figure 10. We also demonstrate that feeding a MCD diet disrupts the normal transport of bile acids and inhibits enzymes involved in bile acid synthesis, which induces cholestasis independent of RXR α . In conclusion, while hepatocyte RXR α deficiency provides resistance against oxidative stress, these data suggest a critical role for RXR α in hepatic fatty acid homeostasis and protection against MCD-induced hepatocyte injury. Studies on how H-RXR α -null mice adapt to complete biliary obstruction using BDL are important in order to define the role of RXR α in providing hepatobiliary protection in the presence of elevated bile acids.

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Abbreviations

L-FABP	liver fatty acid binding protein
Cyp2e1	cytochrome P450 2e1
Cyp4a14	cytochrome P450 4a14
MCD	methionine and choline deficient
PPARα	peroxisome proliferator-activated receptor alpha
SREBP	sterol regulatory element binding protein
ASH	

	alcoholic steatohepatitis
NASH	non-alcoholic steatohepatitis
NAFLD	nonalcoholic fatty liver disease
RT-PCR	reverse transcription-polymerase chain reaction
ALT	alanine aminotransferase
ALP	alkaline phosphatase
LPO	lipid peroxidation
NEFA	nonesterified fatty acid
TBST	Tris-buffered saline with 0.1% Tween 20
ACOX1	acyl-CoA oxidase 1
CAR	constitutive androstane receptor
Oatp	organic anion-transporting polypeptides
Ntcp	sodium/taurocholate-cotransporting polypeptide
Mrp	multidrug resistance-associated protein
Mdr2	multidrug resistance protein 2
Bsep	bile salt export pump
FXR	farnesoid X receptor
MIP-2	macrophage inflammatory protein-2
IL-1β	interleukin-1 β
TNFα	tumor necrosis factor α

α-SMA	α -smooth muscle actin
CPT-1	carnitine palmitoyltransferase 1
ACC-1a	acetyl CoA carboxylase-1a
FAS	fatty acid synthase
FAT/CD36	fatty acid translocase
BDL	bile-duct ligation
VLDL	very low density lipoprotein

Fig.1.

Representative photomicrographs of hematoxylin and eosin (H & E) staining of liver sections in wild type (WT) and hepatocyte RXR α -null (H-RXR α -null) mice fed control or a methionine and choline deficient (MCD) diet.

Male WT or H-RXR α -null mice were fed control (■) or MCD (■) diets for 2 weeks. H & E staining (original magnification $\times 400$) from different treatment groups was performed as described in Materials and Methods. In the livers of mice fed the MCD diet, H & E staining revealed steatosis in both WT and H-RXR α -null mice fed a MCD diet.

Fig. 2.

mRNA expression of hepatic transcription factors and lipid synthesizing genes in wild type (WT) and hepatocyte RXR α -null (H-RXR α -null) fed a control or a methionine and choline deficient (MCD) diet.

Male WT or H-RXR α -null mice were fed a control (■) or a MCD (▒) diets for 2 weeks. Total hepatic RNA was isolated and mRNA levels were determined by real-time PCR as described in Materials and Methods. Data represent mean $\pm$ SEM (n = 4-5). Pound (#) represent statistically significant difference ($P < 0.05$) between mice fed control and MCD diet. Daggers (†) represent statistically significant difference ($P < 0.05$) between WT mice and H-RXR α -

null mice fed the MCD diet. Two-way ANOVA revealed significant effects ($P < 0.05$) of genotype and the MCD diet on expression of FAS, ACC-1a, SREBP-2, and CD-36.

Fig. 3.

Hepatic transcription factor, fatty acid oxidation genes mRNA and protein expression in wild type (WT) and hepatocyte RXR α -null (H-RXR α -null) mice fed a control or a methionine and choline deficient (MCD) diet.

Male WT or H-RXR α -null mice were fed a control (■) or a MCD (▒) diets for 2 weeks. Total hepatic RNA was isolated and mRNA levels were determined by real-time PCR as described in Materials and Methods. Liver homogenate (50 μ g/lane), cytosol (30 μ g/lane) or microsomes (30 μ g/lane) from control or a MCD-fed mice was electrophoresed on 10 or 15% SDS-polyacrylamide gels, transferred to polyvinylidene difluoride (PVDF) membranes, and incubated with anti-ACOX1, L-FABP or Cyp4a14 antibody. Following probing, blots were

stripped and reprobed with anti- β -actin or Gapdh antibody. Data represent mean $\pm$ SEM (n = 3-5). Asterisks (*) represent statistically significant difference ($P < 0.05$) between WT and H-RXR α -null mice. Pound (#) represent statistically significant difference ($P < 0.05$) between mice fed a control and a MCD diet. Daggers (†) represent statistically significant difference ($P < 0.05$) between WT mice and H-RXR α -null mice fed the MCD diet. Two-way ANOVA revealed significant effects ($P < 0.05$) of genotype and the MCD diet on expression of CPT-1 and Cyp4a14.

Fig. 4.

MIP-2, TNF α , IL-1 β , α -SMA mRNA and IL-1 β protein levels in wild type (WT) and hepatocyte RXR α -null (H-RXR α -null) mice fed a control or a methionine and choline deficient (MCD) diet.

Male WT or H-RXR α -null mice were fed control (■) or MCD diets (■) for 2 weeks. MIP-2 (a), TNF α (b), IL-1 β (c), and α -SMA (d) normalized to Gapdh or β -actin mRNA levels were determined by real-time PCR. Liver homogenate (50 μ g/lane) from control or a MCD-fed mice was electrophoresed on 15% SDS-polyacrylamide gel, transferred to polyvinylidene difluoride (PVDF) membranes, and incubated with anti-IL-1 β antibody. Following probing, blots were stripped and reprobed with anti-gapdh antibody. Data represent mean $\pm$ SEM (n = 4). Asterisks

(*) represent statistically significant difference ($P < 0.05$) between WT and H-RXR α -null mice. Pound (#) represent statistically significant difference ($P < 0.05$) between mice fed a control and a MCD diet. Daggers (†) represent statistically significant difference ($P < 0.05$) between WT mice and H-RXR α -null mice fed the MCD diet. Two-way ANOVA revealed significant effects ($P < 0.05$) of genotype and the MCD diet on gene expression of MIP-2, α -SMA, and IL-1 β .

Fig. 5.

Hepatic Cyp7a1 and Cyp8b1 mRNA levels in wild type (WT) and hepatocyte RXRα-null (H-RXRα-null) mice fed a control or a methionine and choline deficient (MCD) diet.

Male WT or H-RXRα-null mice were fed control (■) or MCD (▒) diets for 2 weeks. Cyp7a1 and Cyp8b1 mRNA levels were determined by real-time PCR. Data represent mean ± SEM (n = 4-5). Asterisks (*) represent statistically significant difference ($P < 0.05$) between WT and H-RXRα-null mice. Pound (#) represent statistically significant difference ($P < 0.05$) between mice fed a control and a MCD diet. Two-way ANOVA revealed no significant effects of genotype and the MCD diet on gene expression of Cyp7a1 and Cyp8b1.

Fig. 6.

Hepatic uptake transporters Ntcp, Oatp1a1, Oatp1a4, and Oatp1b2 mRNA levels in wild type (WT) and hepatocyte RXRα-null (H-RXRα-null) mice fed a control or a methionine and choline deficient (MCD) diet.

Male WT or H-RXRα-null mice were fed control (■) or MCD (■) diets for 2 weeks. Ntcp, Oatp1a1, Oatp1a4, and Oatp1b2 mRNA levels were analyzed by branched DNA (bDNA) signal amplification assay as described in Materials and Methods. Data represent mean ± SEM (n = 4-5). Pound (#) represent statistically significant difference ($P < 0.05$) between mice fed a control and a MCD diet. Daggers (†) represent statistically significant difference ($P < 0.05$) between WT mice and H-RXRα-null mice fed the MCD diet. Two-way ANOVA revealed significant effects ($P < 0.05$) of genotype and the MCD diet only on gene expression of Oatp1a4.

Fig. 7.

Hepatic canalicular efflux transporters Bsep, Mrp2, Mdr2, and Mdr1b mRNA levels in wild type (WT) and hepatocyte RXRα-null (H-RXRα-null) mice fed control or methionine and choline deficient (MCD) diet.

Male WT or H-RXRα-null mice were fed control (■) or MCD (▒) diets for 2 weeks. Bsep, Mrp2, Mdr2, and Mdr1b mRNA levels were analyzed by branched DNA (bDNA) signal amplification assay as described in Materials and Methods. Data represent mean $\pm$ SEM ($n = 4-5$). Asterisks (*) represent statistically significant difference ($P < 0.05$) between WT and H-RXRα-null mice. Daggers (†) represent statistically significant difference ($P < 0.05$) between WT mice and H-RXRα-null mice fed the MCD diet. Two-way ANOVA revealed no significant effects of genotype and the MCD diet on hepatic canalicular efflux transporter gene expression.

Fig. 8.

Hepatic basolateral efflux transporters Mrp3 and Mrp4 mRNA levels in wild type (WT) and hepatocyte RXR α -null (H-RXR α -null) mice fed a control or a methionine and choline deficient (MCD) diet.

Male WT or H-RXR α -null mice were fed control (■) or MCD (▒) diets for 2 weeks. Mrp3 and Mrp4 mRNA levels were analyzed by branched DNA (bDNA) signal amplification assay as described in Materials and Methods. Data represent mean $\pm$ SEM (n = 4-5). Asterisks (*) represent statistically significant difference ($P < 0.05$) between WT and H-RXR α -null mice. Pound (#) represent statistically significant difference ($P < 0.05$) between mice fed a control and a MCD diet. Daggers (†) represent statistically significant difference ($P < 0.05$) between

WT mice and H-RXR α -null mice fed the MCD diet. Two-way ANOVA revealed no significant effects of genotype and the MCD diet on Mrp3 and Mrp4 gene expression.

Fig.9.

Serum alanine aminotransferase (ALT), alkaline phosphatase (ALP), and bile acid levels in wild type (WT) and hepatocyte RXR α -null (H-RXR α -null) mice fed control or a methionine and choline deficient (MCD) diet.

Male WT or H-RXR α -null mice were fed control (■) or MCD (▒) diets for 2 weeks. Serum ALT, ALP, and bile acids were determined as described in Materials and Methods. Data represent mean $\pm$ SEM (n = 5-8). Asterisks (*) represent statistically significant difference ($P < 0.05$) between WT and H-RXR α -null mice. Pound (#) represent statistically significant difference ($P < 0.05$) between mice fed control and MCD diet. Daggers (†) represent statistically significant difference ($P < 0.05$) between WT mice and H-RXR α -null mice fed the

MCD diet. Two-way ANOVA revealed significant effects ($P < 0.05$) of genotype and the MCD diet on ALT and ALP levels but not serum bile acid levels.

Fig. 10.

Hepatocyte RXR α -deficiency causes dysregulation of fatty acid synthesis and oxidation. MCD diet ingestion activates the sterol regulatory element binding proteins (SREBP)-regulated genes (FAS, ACC-1a and HMG CoA reductase) which up-regulate fatty acids, hepatic lipids and bile acid synthesis. Inactivation of PPAR α and PPAR α -target genes including CPT-1, ACOX1, and L-FABP by MCD diet and a lack of Cyp4a14 gene induction in the H-RXR α -null mice produces impaired fatty acid β -oxidation and hepatic fatty acid overload. Free fatty acid accumulation may lead to proinflammatory cytokine gene activation and liver injury in H-RXR α -null mice.

Table 1

Sequences of primers and probes used for real-time quantitative PCR.

Name		Sequence	Accession #
MIP-2	Sense	GAACATCCAGAGCTTGAGTGTGA	NM 009140
	Antisense	CCCTTGAGAGTGGCTATGACTTC	
	Probe	AGGACCCCACTGCGCCAGAG	
TNF-1 α	Sense	ACAAGGCTGCCCCGACTAC	NM013693
	Antisense	TTTCTCCTGGTATGAGATAGCAAATC	
	Probe	TGCTCCTCACCCACACCGTCAGC	
α -SMA	Sense	CCTGACGGGCAGGTGATC	NM 007392
	Antisense	ATGAAAGATGGCTGGAAGAGAGTCT	
	Probe	CGAACGCTTCCGCTGCCCA	
CD36	Sense	TCCAGCCAATGCCTTTGC	NM007643
	Antisense	TGGAGATTACTTTTTCAGTGCAGA A	
	Probe	TCACCCCTCCAGAATCCAGACAACCA	
FAS	Sense	CCCGGAGTCGCTTGAGTATATT	NM007988
	Antisense	GGACCGAGTAATGCCATTGAG	
	Probe	AGCCCATGGCACGGGCACC	
L-FABP	Sense	TGCATGAAGGGAAGAAAATCAAA	NM017399
	Antisense	CCCCCAGGGTGAACCTCATT	
	Probe	TCACCATCACCTATGGACCCAAAGTGG	
ACC1	Sense	ATGTCCGCACTGACTGTAACCA	NM133360
	Antisense	TGCTCCGCACAGATTCTTCA	
	Probe	TCCTCAACTTTGTGCCACGGTCA	
β -Actin	Sense	CTTCTTTGCAGCTCCTTCGTTG	NM 007393
	Antisense	CGACCAGCGCAGCGATATC	
	Probe	CCACACCCGCCACCAGTTCGCC	
GAPDH	Sense	TGTGTCCGTCGTGGATCTGA	NM 001001303
	Antisense	CCTGCTTCACCACCTTCTTGA	
	Probe	CCGCCTGGAGAAACCTGCCA	
IL-1 β	Sense	AAGATGAAGGGCTGCTTCCA	NM008361
	Antisense	GTGCTGCTGCGAGATTTGAA	
	Probe	CCTTTGACCTGGGCTGTCCTGATGA	
CD36	Sense	TCCAGCCAATGCCTTTGC	NM007643
	Antisense	TGGAGATTACTTTTTCAGTGCAGA A	
	Probe	TCACCCCTCCAGAATCCAGACAACCA	
CPT-1	Sense	CGATCATCATGACTATGCGTACT	NM013495
	Antisense	GCCGTGCTCTGCAAACATC	
	Probe	CTGAAGGTGCTGCTCTCCTACCATTCA	
SREBP-1c	Sense	CATGCCATGGGCAAGTACAC	NM011480
	Antisense	TGTTGCCATGGAGATAGCATCT	
	Probe	AACCTGGCACTAAGTGCCCTCAACCTG	
SREBP-2	Sense	ATGATACCCCGACGTTTCA	NM 033218
	Antisense	GCTGCGTTCTGGTATATCAAAGG	

Name		Sequence	Accession #
PPAR α	Probe	CGCTCCGCAGACGAGGATCATCC	NM011144
	Sense	GATTCAGAAGAAGAACCGGAACA	
	Antisense	TGCTTTTTCAGATCTTGGCATTG	
HMGCoA-Reductase	Probe	TCTGTCGGGATGTACACAATGCAATTC	NM008255
	Sense	GGCAGTCAGTGGGAACTATTGC	
	Antisense	CAGTCTTTCCTCGTCCTTCGA	
Cyp7a1	Probe	CCGACAAGAAGCCTGCTGCCATAAAC	NM007824
	Sense	CCATGATGCAAAACCTCCAAT	
	Antisense	TGACCCAGACAGCGCTCTTT	
Cyp8b1	Probe	TGTCATGAGACCTCCGGGCCTTCC	NM010012
	Sense	GCCCTTACTCCAAATCCTACCA	
	Antisense	TCGCACACATGGCTCGAT	
ACOX1	Probe	TCAGACTCCAGGGATGTTGCTCAATGG	NM 015729
	Sense	TTTGTGTGCCCTATCCGTGAGA	
	Antisense	GCCGATATCCCCAACAGTGA	
Cyp4a14	Probe	TGGGACCCACAAGCCTCTGCCA	NM 007822
	Sense	CAAGACCCTCCAGCATTTCC	
	Antisense	GGTGTGGCAAGGCATTCC	
	Probe	TCACCCGCTCTTTACCTTCCTG	

Table 2

Effect of methionine and choline deficient diet (MCD) diet on body weight, liver weight, biochemical parameters, and liver pathology.

Parameter	Wild type		RXR α KO	
	Control	MCD	Control	MCD
Body weight (g)	34.6 $\pm$ 1.0	27.8 $\pm$ 1.4 [#]	32.5 $\pm$ 0.9	24.2 $\pm$ 0.9 ^{#, †}
Absolute liver weight (g)	1.20 $\pm$ 0.0	0.91 $\pm$ 0.1	1.23 $\pm$ 0.0	0.92 $\pm$ 0.0 [#]
Liver/body weight (%)	3.5 $\pm$ 0.2	3.3 $\pm$ 0.2	3.8 $\pm$ 0.1	3.8 $\pm$ 0.2
Serum cholesterol (mg/dl)	125 $\pm$ 17.9	55 $\pm$ 4.1 [#]	203 $\pm$ 10.1 [*]	87 $\pm$ 7.5 ^{#, †}
Serum triglycerides (mg/dl)	110 $\pm$ 10.7	55 $\pm$ 5.7 [#]	379 $\pm$ 28.5 [*]	91.9 $\pm$ 10.7 ^{#, †}
Hepatic triglyceride (mg/g liver)	6.9 $\pm$ 0.5	9.4 $\pm$ 0.7 [#]	7.72 $\pm$ 0.51	10.0 $\pm$ 0.2 [#]
Hepatic NEFA (μ mol/mg protein)	0.63 $\pm$ 0.06	0.58 $\pm$ 0.08	0.61 $\pm$ 0.04	0.76 $\pm$ 0.02 [#]
TBARS levels (nmol/mg)	4.3 $\pm$ 0.1	6.3 $\pm$ 0.4 [#]	3.3 $\pm$ 0.1 [*]	3.7 $\pm$ 0.3 [†]
Cyp2e1 activity (nmol/mg)	17.8 $\pm$ 2.3	25.5 $\pm$ 1.7 [#]	14.3 $\pm$ 1.3	18.9 $\pm$ 1.7 [†]
Steatosis score	0.0 $\pm$ 0.0	1.6 $\pm$ 0.3 [#]	0.0 $\pm$ 0.0	2.3 $\pm$ 0.7 [#]
Inflammation	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.0 $\pm$ 0.0	0.3 $\pm$ 0.2

Data are mean $\pm$ SEM for 4-8 mice per group.

* $P < 0.05$, statistically significant difference between WT and H-RXR α -null mice.

[#] $P < 0.05$ statistically significant difference between mice fed a control and a MCD diet.

[†] $P < 0.05$ statistically significant difference between WT mice and H-RXR α -null mice fed a MCD diet. Two-way ANOVA revealed significant effects ($P < 0.05$) of genotype and the MCD diet on serum cholesterol and triglyceride levels.